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(54) Specific binding assays

(57) An analyte is detected in an enzyme-linked assay, wherein the presence of a first enzyme is related to the amount of analyte. The first enzyme converts an inactive precursor of PQQ to PQQ or an active analogue thereof. The PQQ complements an apo-enzyme such as apo-GDH to form an active second enzyme (GDH). The second enzyme catalyses a reaction leading to a detectable signal. This signal is preferably measured bioelectrochemically such as by a GDH electrode as shown in Fig. 1.

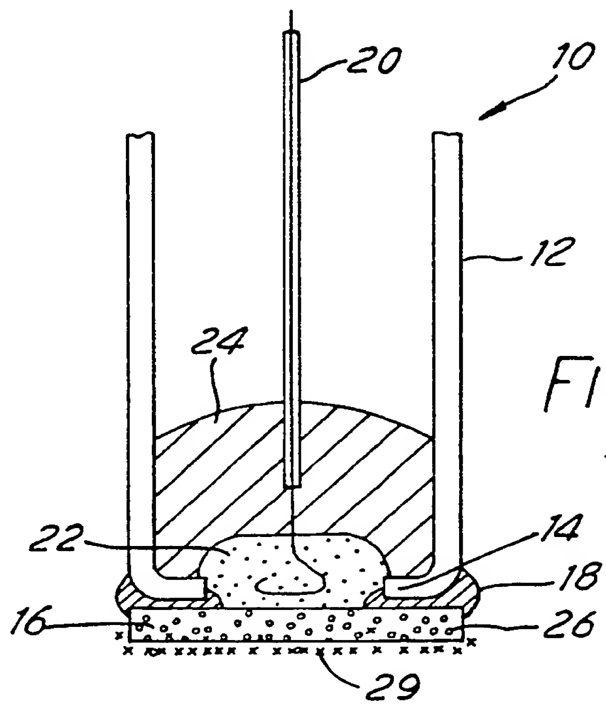

FIG. 1

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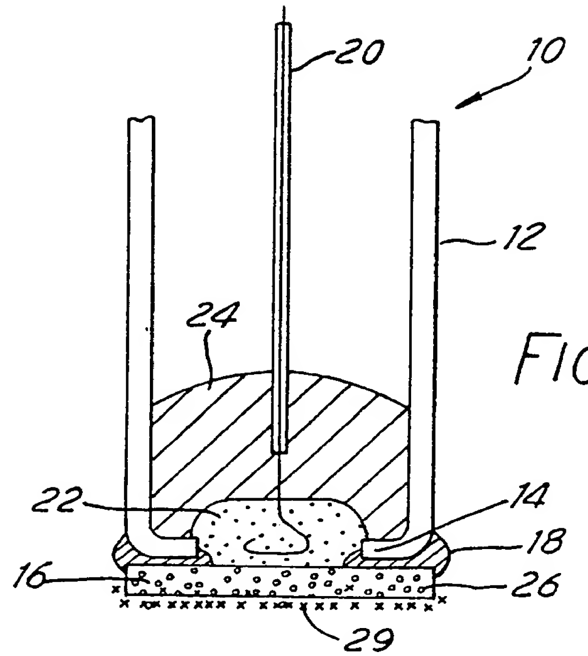
20
10
12
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14
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16
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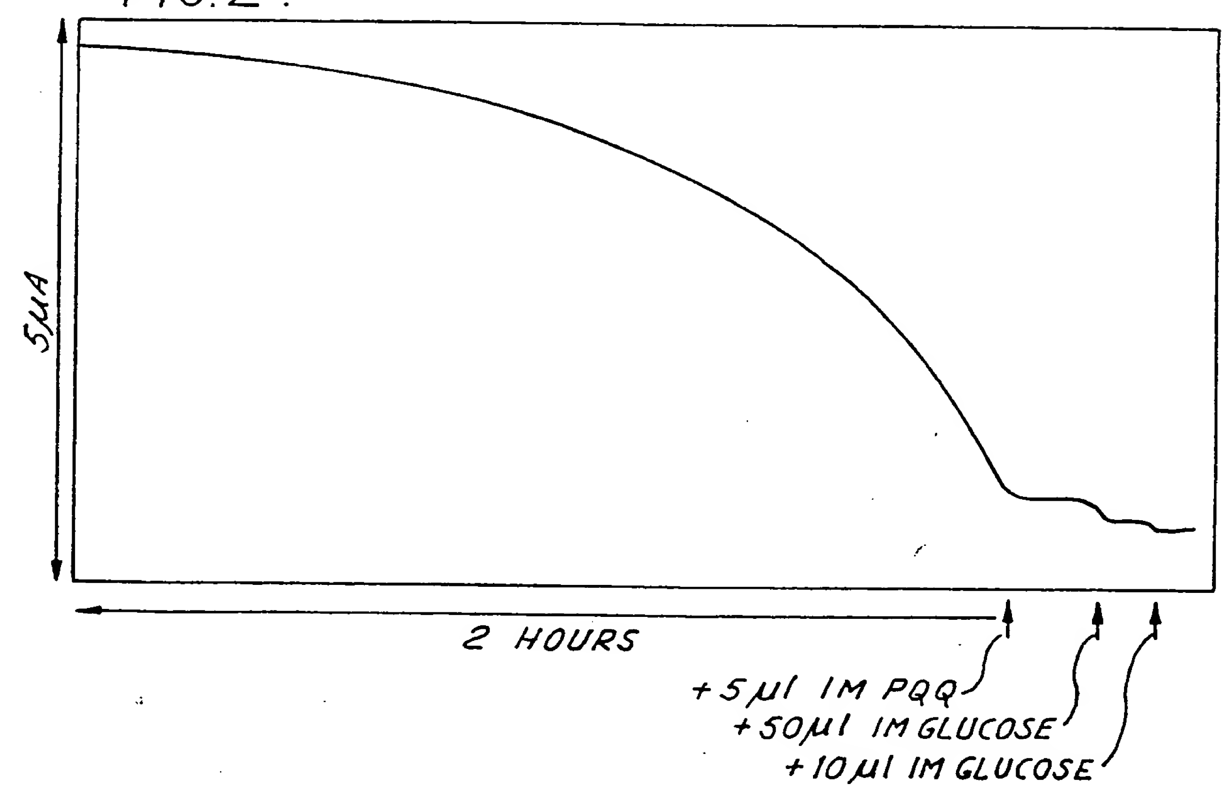
FIG.1

FIG.2.


5μA
2 HOURS
+5μl 1M PQQ
+50μl 1M GLUCOSE
+10μl 1M GLUCOSE

SPECIFICATION

Specific binding assays

The present invention relates to specific binding assays.

The present invention seeks to provide improvements in assays for specific binding wherein the binding is detected by a signal generated by a secondary event dependent on the extent of binding. The signal, of the present invention, is generated by the reconstitution of a holo-enzyme, such as the quinoprotein glucose dehydrogenase, from the apo-enzyme and co-enzyme or prosthetic group.

According to the present invention there is provided a specific binding assay for an analyte which comprises:

(a) binding of a specific binding pair (sbp) to an extent related to the presence of the analyte, which sbp comprises an enzyme;

(b) release of a co-enzyme from a derivative thereof by the action of said enzyme, the derivative being a substrate for said enzyme;

(c) re-constitution of a holo-enzyme from the co-enzyme and an apo-enzyme; and

(d) detection of the holo-enzyme;

wherein the co-enzyme derivative does not combine with the apo-enzyme to reconstitute substantial holo-enzyme activity.

In a one form the co-enzyme is 2,7,9-tricarboxy-1*H*-pyrrolo-(2,3-*f*)quinoline-4,5-dione (PQQ) or an analogue thereof which reconstitutes holo-enzyme activity, and the apo-enzyme is a quinoprotein.

Suitably, the specific binding pair comprises an immobilised binding member specific for an analyte and a second binding member conjugated to an enzyme. Suitably, the second binding member is either specific for the immobilised binding member or the analyte. For example, the second binding member may compete with the analyte for conjugation with the immobilised binding member, or the immobilised binding member may compete with the analyte for conjugation with the second binding member. Alternatively, the analyte may contain a site which binds specifically to the immobilised binding member and a different site which binds specifically to the second binding member.

Generally, the enzymic release of the co-enzyme provides primary amplification. Suitably, the holo-enzyme generates a signal, which is preferably an electrochemical signal though it also may be a spectrophotometric signal. Generally, the action of the holo-enzyme provides secondary amplification.

In order that the present invention may be more clearly understood, it will be further illustrated by way of example only and with reference to the drawings, wherein:

Figure 1 shows a GDH electrode in cross-section; and

Figure 2 is a graph showing a response of a PQQ depleted GDH electrode.

Applications of modified PQQ and apo-GDH in specific binding assays

Bacterial glucose dehydrogenase (GDH) is a member of a new, and increasingly important, quinoprotein class of dehydrogenases (Duine and Frank, 1981). Some alcohol dehydrogenases, some methanol dehydrogenases and some methylamine dehydrogenases are further examples (Duine and Frank, 1981). GDH was originally purified from *Bacterium anitratum* (Hauge, 1960) and it has now been found in a wide variety of gram-negative organisms (van Schie, et al., 1984).

The GDH prosthetic group, 2,7,9-tricarboxy-1*H*-pyrrolo(2,3-*f*)-quinoline-4,5-dione (pyrroloquinoline quinone) (PQQ), was identified by two independent research groups (Salisbury, et al., 1979; Duine, et al., 1980), and has been synthesised by a number of routes (Corey and Tramontano, 1981; Gainor and Weinreb, 1981; Hendricksen and de Vries, 1985; MacKenzie, et al., 1983; Buchi, et al., 1985). Apo-GDH (GDH from which PQQ has been removed) from *Acinetobacter calcoaceticus, Pseudomonas aeruginosa* and *Klebsiella aerogenes* strains has been shown to act as a highly specific biological test for PQQ (Duine, et al., 1983). The relationship between GDH activity and PQQ concentration was linear between 2 and 60 nM, and PQQ adducts had negligible activity in this test (Duine, et al., 1983).

PQQ forms addition compounds with quinoprotein substrates, activators and stabilisers e.g. alcohols, amines and cyanide (Dekker, et al., 1982). The chemical and electrochemical properties of a number of derivatives and analogues of PQQ have been investigated in detail (Sleath, et al., 1985). The close analogue of PQQ, 7,9-didecarboxy PQQ, was shown to possess electrochemical and dynamic properties which were virtually indistinguishable from those of PQQ, but it was found to have no discernable activity with apo-GDH. However, 4,7-phenanthroline-5,6-dione, which also had similar electrochemical properties to PQQ (Eckert and Bruice, 1983) was found to produce activity with apo-GDH (Conlin, et al., 1985). However, in the reconstitution assay it was found to produce thirty times less activity than synthetic PQQ. Duine, et al (1980) demonstrated

that a range of phenanthroline-diones and PQQ derivatives which were very closely related to PQQ were unable to restore the activity to apo-GDH on reconstitution. Another derivative of PQQ, the trimethyl ester (PQQTME), was synthesised (Corey and Tramontano, 1981) and used to catalyse the oxidation of alcohols in a non-enzymic system. However, this compound was not investigated for biological activity with apo-GDH.

Thus, PQQ and a variety of closely related compounds have been synthesised. Most of these have been tested for biological activity with apo-GDH but only one has shown any activity, and this was thirty times lower than that of PQQ.

The homogeneous kinetics and enzymic mechanism of GDH have been described (D'Costa, et al., 1984; D'Costa, 1986). Second order rate constants for the reduction of a number of electrochemical mediators by soluble GDH have also been determined using direct current cyclic voltammetry (D'Costa, et al., 1986). In addition, GDH has been immobilised on a porous graphite electrode in the presence of 1,1'-dimethylferrocene to produce a biosensor for glucose (D'Costa, et al., 1986). Furthermore, a GDH-based biosensor which had been made partially deficient in PQQ produced a six-fold increase in current over a period of two hours following the addition of 500 nM PQQ. In free solution the rate of re-association of PQQ with apo-GDH can be expected to occur more rapidly (Conlin, et al., 1985).

One example of a specific binding assay is the enzyme-linked immunosorbent assay (ELISA). One form of the ELISA technique, involving a competitive assay using antigen-enzyme conjugate and immobilised antibody, comprises the following steps.

Immobilisation of antibody to solid phase,

(ii) wash,

(iii) incubate with enzyme-labelled antigen in the presence or absence of standard or sample antigen,

(iv) wash,

(v) incubate with enzyme substrate and determine the presence of product of the enzymatic reaction.

In accordance with the present invention, the enzyme substrate in (v) is a modified enzymatically inactive precursor of PQQ, which is converted by the label enzyme to PQQ or an enzymatically active analogue thereof, which is the prosthetic group of secondary apo-enzyme, which is thereby converted to the active holo-enzyme to produce the detectable signal.

From this it can be seen that the concentration of enzyme product (PQQ or active analogue) varies inversely with the concentration of the analyte being measured.

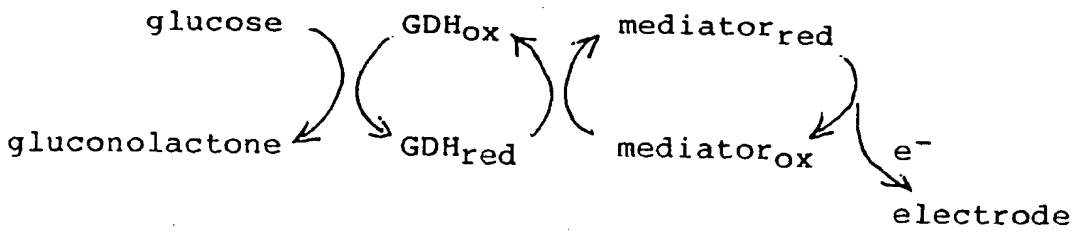
Examples of sbp's which may be used are given in Table 1 below.

Table 1: Examples of Specific Binding Pairs

Ligand	*Anti-ligand*
antigen	specific antibody
antibody	antigen
hormone	hormone receptor
hormone receptor	hormone
polynucleotide strand	complementary strand
avidin	biotin
biotin	avidin
protein A	immunoglobulin
immunoglobulin	protein A
enzyme	enzyme cofactor/substrate
enzyme cofactor/substrate	enzyme
lectins	carbohydrate
carbohydrate	lectins
affinity dye	protein
protein	affinity dye

In conventional ELISA techniques the enzyme label is usually one that yields a chromogenic product in a signal-producing system, the concentration of which can be determined spectrophotometrically. In the present example, the primary reaction product (PQQ or active analogue) is measured by the extent to which GDH activity is restored by reconstitution of the holoenzyme. GDH activity is preferably m asured electrochemically though it may be measured spectrophotometrically.

An amperometric assay for GDH is shown below.



Electrons are passed from glucose to GDH and then to an electrode by way of a mediator. The enzyme may either be in free solution with a soluble mediator or co-immobilised with a mediator on an electrode surface as shown in Fig. 1. In the example given below, a ferrocene derivative is used as the mediator. However, a variety of other mediators may used, as may conducting organic salts such as tetrathiafulvalene-tetraquinodimethane (TTF–TCNQ), or either of TTF or TCNQ alone.

Construction of graphite electrodes

Referring to Fig. 1, a GDH electrode 10 was constructed in the following manner:

(i) A 2.5 cm length of 6 mm outside diameter glass tubing 12 was heated at one end 14, turned in, and flattened off without closing the tubing.

(ii) A disc 16 (6 mm diameter) was punched out of a 1 mm thick strip of porous graphite (Union Carbide, Ohio, USA) and washed twice in acetone (30 minutes for each wash with gentle agitation), once in boiling distilled water (1 hour), and dried in an air oven at 100°C.

(iii) The graphite disc 16 was glued to the modified end of the glass tubing 12 by the rim using Araldite (Ciba-Geigy Trade Mark) epoxy resin 18.

(iv) Electrical contact to the disc 16 was made by cementing to it a length of 0.2 mm insulated wire 20 using a drop of electrically conducting (silver-loaded) epoxy resin 22 (Johnson Matthey Chemicals Ltd., Royston, Herts., UK). Insulation of the electrical contact was made by covering it with an insulating resin 24 comprising a mixture of 9 parts epon resin (grade 815) and 1 part triethylenetetramine catalyst 24 (both obtained from Polysciences Inc., Warrington PA, USA) and allowing it to set at 60°C in an oven overnight.

(v) The electrode 10 was tested before use for electrical conductivity between the graphite surface 16 and the terminal wire 20.

Immobilisation of GDH onto graphite using water-soluble carbodiimide

The graphite electrode 10 was supported in an inverted position while a 5 μl drop of 1,1'-dimethylferrocene/toluene solution (20 mg ml $^{-1}$) was placed on the graphite surface 16 and allowed to dry in air. This immobilises ferrocene intermediate 26 on the graphite surface. After 30 minutes at room temperature the electrode 10 was suspended vertically with the grafoil tip 16 pointing downwards, just into the surface of a 20 mg ml $^{-1}$ solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-p-toluenesulphonate (Sigma Chemical Company) in 200 mM sodium acetate buffer, pH 4.5 for 80 minutes at room temperature. The electrode 10 was washed by immersing the tip in 20 mM sodium acetate buffer, pH 5.5 and then suspended in 1.5 ml ice-cold GDH stock solution to immobilise GDH 28 on the electrode 10. The electrode was then immersed in a high concentrate phosphate buffer which caused the PQQ to leak out of the holo-enzyme leaving apo-GDH 28 on the electrode 10. After five hours the electrode 10 was washed as before then stored in 200 μl sodium buffer, pH 7.0 at 4°C for up to five weeks before use.

A GDH electrode made as above was used in a three-electrode system and was set at 160 mV against the standard calomel electrode. The assay was carried out in 10 mM phosphate, pH 7.0 at 28°C and in a final volume 10 ml.

Fig. 2 shows the response obtained from a PQQ depleted GDH electrode. The electrode was allowed to settle down to a low current of about 0.5 to 1 μA. Addition of 10 μ1 1M glucose produced a minute rise, and addition of 50 μ1 1mM glucose a further small rise. Addition of 5 μl 1mM PQQ produced a large response, causing the current to rise from about 1 μA to about 5 μA, and taking about 2 hours to settle down.

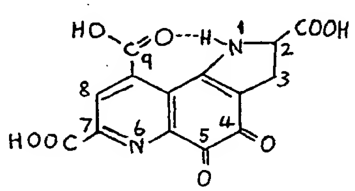
This experiment shows the sensitivity of the electrode to very small amounts of PQQ (here 5 μl 1mM PQQ diluted into 10 ml).

Further the comparatively slow reassociation kinetics displayed by the immobilised enzyme compared to that occurring in solution means that quantitative rate measur ments can be made as opposed to measuring the extent of reactivation.

Additions of PQQ to blank or normal GDH electrodes produced no response, while additions of glucose to normal GDH electrodes produced normal current increases.

Modification of PQQ

The structure of PQQ is shown below.

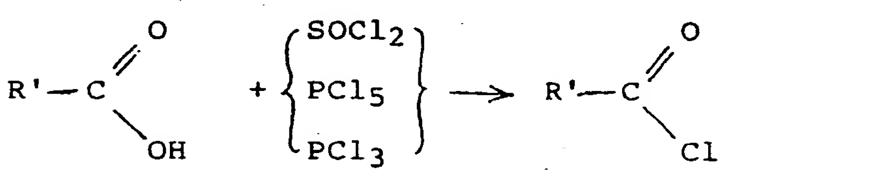


The C2, C7 and C9 carboxylic acid groups are potential sites for PQQ modification. Whilst not of catalytic importance the addition of bulky groups onto all or any of the carboxyl carbon atoms could prevent prosthetic group binding to apo-GDH due to steric hindrance. Such modification might also remove the negative charge. Therefore, If one or more of these groups is important in the binding of the prosthetic group to the active-site of GDH, the modification might abolish or seriously impair the binding interaction.

Reactive derivatives of carboxylic acid groups can be readily prepared e.g. acid chlorides, anhydrides, etc., which will undergo reaction with nucleophilic compounds such as amines or alcohols to yield amide and ester linkages respectively. An alternative method of modifying the carboxyl groups of PQQ would be the use of compounds such as carbodiimides. These activate carboxyl groups which are then able to undergo condensation reactions with amino and hydroxyl groups, again yielding amide and ester linkages. Enzymes which are capable of cleaving such bonds include the hydrolases (EC 3.1.). General reaction schemes are shown below.

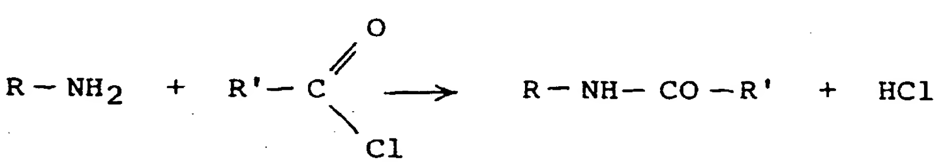
Examples of possible modifications to PQQ

A. *Preparation of PQQ acid chloride*

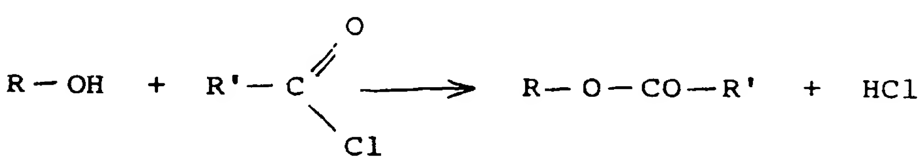


PQQ PQQ acid chloride

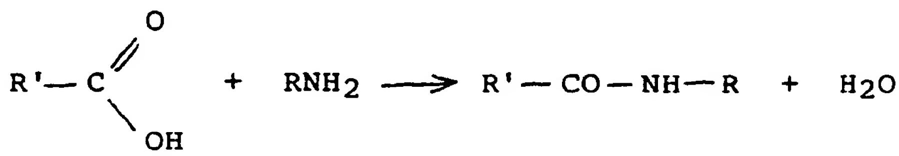
B. *Reaction of an acid chloride with:*
(i) an amine



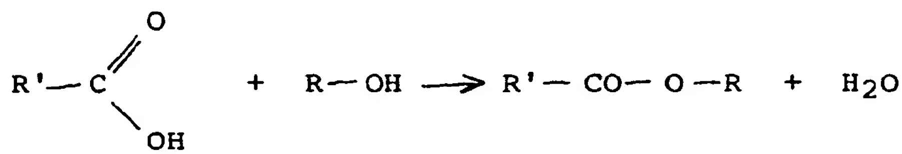
(ii) an alcohol



C. *Carbodiimide initiated condensation reactions*
(i) Acylation of amines

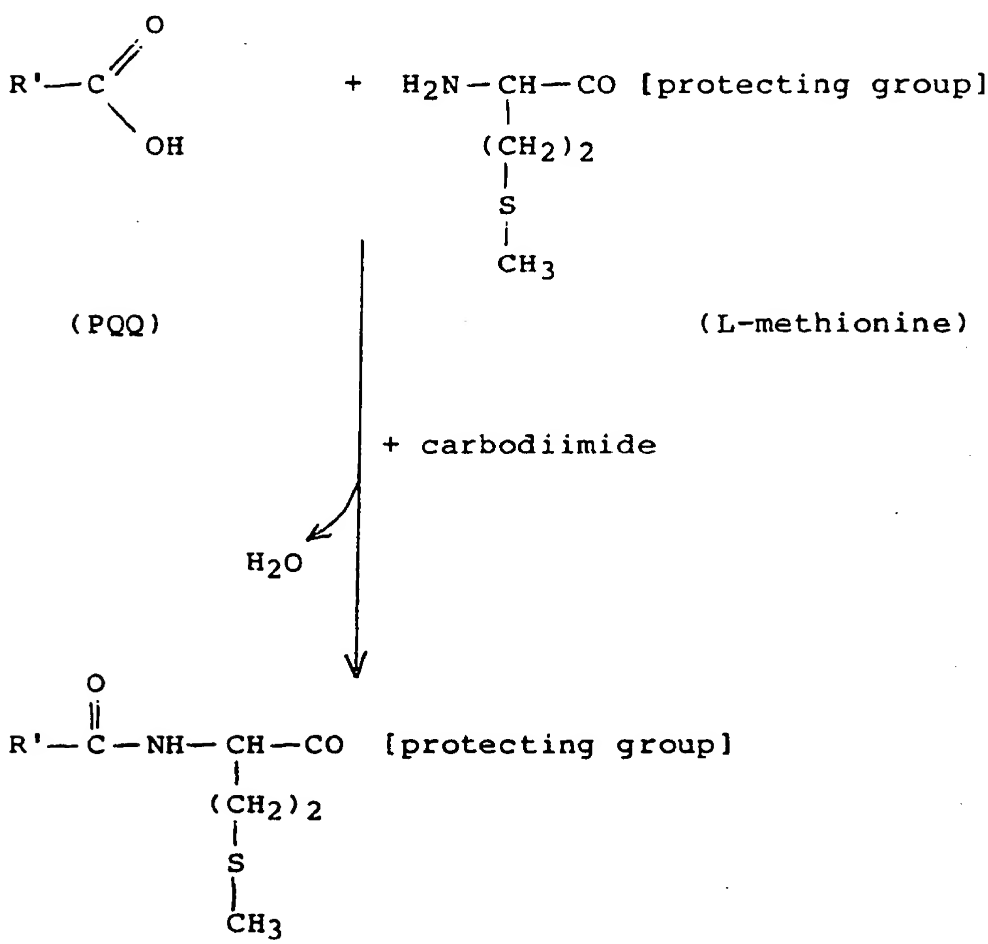


(ii) Esterification with hydroxy groups



Since acid chlorides are highly reactive species, it is sometimes necessary to prevent PQQ acid chloride molecules from reacting with each other. This can be achieved by chemically "protecting" any susceptible groups or alternatively by immobilising it on a solid phase prior to the modification reactions to ensure that PQQ interactions cannot occur.

The nature of the R group will depend on the specificity of the enzyme chosen as the primary amplification label. For example, aminoacylase (EC 3.5.1.14) will cleave amide bonds of N-acyl amino acids. Thus PQQ which has been modified with, for example, L-methionine (as shown below) might serve as a substrate for this enzyme, the activity of which would yield native PQQ provided that the nature of the R' group (PQQ) is not a critical factor in substrate binding.



When preparing the L-methionine derivative of PQQ, the carboxyl group of the methionine is protected, so as to prevent condensation of amino acid molecules with each other.

The above represents only one of the possible routes to achieving a derivative. Alternatives include modifying the orthoquinone moiety of PQQ which has been shown to be essential for activity or the preparation of analogues by, for example, synthetic pathways.

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CLAIMS

1. A specific binding assay for an analyte which comprises:

(a) binding of a specific binding pair (sbp) to an extent related to the presence of the analyte, which sbp comprises an enzyme;

(b) release of a co-enzyme from a derivative thereof by the action of said enzyme, the derivative being a substrate for said enzyme;

(c) re-constitution of a holo-enzyme from the co-enzyme and an apo-enzyme; and

(d) detection of the holo-enzyme;

wherein the co-enzyme derivative does not combine with the apo-enzyme to reconstitute substantial holo-enzyme activity;

said coenzyme being 2,7,9-tricarboxy-1*H*-pyrrolo-(2,3-*f*)-quinoline-4,5-dione (pyrroloquinoline quinone) (PQQ) or an analogue thereof which reconstitutes holo-enzyme activity, and the apo-enzyme is a quinoprotein.

2. An assay according to claim 1 wherein the specific binding pair comprises an immobilised binding member specific for an analyte and a second binding member conjugated to an enzyme.

3. An assay according to claim 2 wherein the second binding member is either specific for

the immobilised binding member or the analyte.

4. An assay according to claim 3 wherein the second binding member competes with the analyte for conjugation with the immobilised binding member, or the immobilised binding member competes with the analyte for conjugation with the second binding member.

5. An assay according to claim 3 wherein the analyte contains a site which binds specifically to the immobilised binding member and a different site which binds specifically to the second binding member.

6. An assay according to claim 1 substantially as described herein.

7. A kit for an assay of any one of the preceding claims.

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